CDC20 is a potential target gene to inhibit the tumorigenesis of MDCK cells

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December 10, 2022

Abstract

MDCK is the main cell line for influenza vaccine production. Previous studies have reported that MDCK cells have tumorigenic ability in nude mice. Although complete cell lysis can be ensured during vaccine production, more caution is needed in vaccine production for human use. Therefore, the use of gene editing technology to establish cells that cannot form tumors can significantly improve the biosafety of influenza vaccines. The key is to understand the genes and molecular mechanisms that affect the tumorigenic ability of MDCK cells. However, our understanding is still superficial. We previously obtained a cell line CL23 with significantly reduced cell proliferation, migration, and invasion through a monoclonal cell screen, and subsequent tumor-bearing experiments in nude mice showed no tumorigenic cells. DIA proteomics method was used to compare the protein expression differences between wild-type (M60) and non-tumorigenic (CL23) cells, and to explore the genes related to tumorigenesis in MDCK cells. The differentially expressed proteins were verified at the mRNA level by RT-qPCR, and several genes involved in cell tumorigenesis were preliminarily screened. Western blot further confirmed that the expression of related proteins was significantly reduced in non-tumorigenic cells. Inhibition of CDC20 expression by RNAi significantly reduced the proliferation and migration of MDCK cells and increased the proliferation of influenza virus. Therefore, CDC20 is an effective target gene for inhibiting the tumorigenicity of MDCK cells. This study lays the foundation for the establishment of target gene screening in genetically engineered non-tumorigenic MDCK cell lines.

CDC20 is a potential target gene to inhibit the tumorigenesis of MDCK cells

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Abstract: MDCK is currently the main cell line used for influenza vaccine production. Previous studies have reported that MDCK cells possess tumorigenic ability in nude mice. Although complete cell lysis can be ensured during the vaccine production process, greater caution is needed regarding the production of human vaccines. Therefore, the use of gene editing to establish cells incapable of forming tumors can significantly improve the biosafety of influenza vaccines. The key is knowledge regarding the genes and molecular mechanisms that affect the tumorigenic ability of MDCK cells; however, our understanding remains superficial. Through monoclonal cell screening, we previously obtained a cell line, CL23, possessing significantly reduced cell proliferation, migration, and invasion abilities, and subsequent tumor-bearing experiments in nude mice showed the absence of tumorigenic cells. With a view to exploring the tumorigenesis-related genes in MDCK cells, DIA proteomics was used to compare the differences in protein expression between wild-type (M60) and non-tumorigenic (CL23) cells. Differentially expressed proteins were verified at the mRNA level by RT-qPCR, and a number of genes involved in cell tumorigenesis were preliminarily screened. Immunoblotting further confirmed that related protein expression was significantly reduced in non-tumorigenic cells. Inhibition of CDC20 expression by RNAi significantly reduced the proliferation and migration of MDCK cells and increased the proliferation of influenza virus; therefore, CDC20 was preliminarily determined as an effective target gene for inhibiting cell tumorigenicity. These results contribute to a more comprehensive understanding of the mechanism underlying cell tumorigenesis and provide a basis for the establishment of target gene screening in genetically engineered non-tumorigenic MDCK cell lines.

Keywords: MDCK, Flu vaccine, Nodular, Proteomics

1. Introduction

Influenza is an infectious respiratory disease caused by the influenza virus. It is highly contagious and easily leads to a pandemic, which seriously threatens human health and socioeconomic development. Influenza vaccination is the most effective means of preventing and controlling an influenza virus pandemic. At present, inactivated viral vaccines produced by animal cells are widely used for the prevention of a variety of infectious diseases. In comparison with the traditional chicken embryo production process, the main advantages are good quality control, ease of scaling-up, and automatic production. In addition, the vaccine virus strain has a low mutation rate in cells, and the antigenicity of the vaccine is closer to that of the natural epidemic strain. The use of a relatively closed bioreactor system can effectively reduce the risk of microbial contamination. Cell-based vaccines do not contain ovalbumin, which reduces the risk of anaphylaxis after vaccination[1] [2] [3]. The Madin–Darby canine kidney (MDCK) cell line has become a good substrate for the amplification of a variety of viruses due to its high viral infection efficiency, rapid proliferation, and low resistance to mutation. In particular, MDCK is recognized as the main cell line for the production of influenza A and B viral vaccines. Inactivated influenza vaccines produced by MDCK cells have been approved in many countries around the world; however, similar to many passaged cell lines, MDCK cells pose the problem of tumor formation in nude mice[4]. Despite sufficient lysis and removal of stromal cells during the vaccine manufacturing process, there remains certain controversy regarding the biological safety of MDCK cells; therefore, increased caution is required when using MDCK cells in the production of human vaccines.

It is particularly important to obtain a cell line incapable of forming tumors to ensure greater safety during...
vaccine production. At present, cells incapable of forming tumors can be obtained by monoclonal strain screening or gene editing; however, the workload for monoclonal cell screening is huge and the success rate is low. Moreover, the cells obtained from screening may have other phenotypes that are not conducive to vaccine production. Gene editing is highly targeted but the main genes and molecular mechanisms underlying tumor formation by MDCK cells must be known. Several signaling pathways have been shown to play an important regulatory role in the process of tumor formation. TNF-α-inducible protein-2 (TNFAIP2) is upregulated in tumor cells cultured in the presence of necrosis factor-α. TNFAIP2 knockout significantly reduces the migration and invasion abilities of nasopharyngeal carcinoma HK1 cells\[^5\] \[^6\]. Among the possible causes of tumorigenesis, the abnormal regulation of cell proliferation is the key link, in addition to the cell cycle-related proteins that play an important role. Studies have shown that CDC20 (cell division cycle protein 20) is a co-activator of the promoting complex/ring body (APC/C) and is therefore involved in mitotic regulation. Studies have shown that the expression level of the CDC20 gene is significantly increased in bladder, breast, colon, rectal, stomach, esophageal, and head and neck cancers. In addition, the expression of CDC20 is significantly positively correlated with the clinical stage of various cancers such as breast, renal, and lung cancers\[^7\]. It has been reported that CDC20 is involved in the occurrence and development of human cutaneous squamous cell carcinoma via the Wnt/β-catenin signaling pathway\[^8\]. At present, research on the mechanisms of cell tumorigenesis mainly focuses on human cells and model organisms, with mechanisms varying greatly among different species, different cells, and even cells in the same population. Little is known regarding the tumorigenic genes or molecular mechanisms in MDCK cells used in vaccine production.

In our previous work, MDCK cells numbered CCL-34TM by ATCC were built and stored as M60. By screening a large number of monoclonal cells, a cell line possessing slow proliferation and low colony rates was found and numbered CL23. In vitro and in vivo tumorigenicity analysis demonstrated that the proliferation, migration, and invasion abilities of these cells were significantly reduced and no tumors formed in nude mice. Moreover, the virus proliferation level in CL23 cells was not significantly affected as compared with that in wild-type M60 cells. Preliminary evaluation of this cell line is of great significance for improving the biosafety of MDCK cell-based inactivated influenza vaccines. To further explore the key proteins and molecular mechanisms that may trigger tumorigenesis in MDCK cells, the DIA quantitative proteomics method can efficiently determine relatively low-abundance proteins in complex samples, greatly improving the credibility of quantitative analysis. In comparison with the traditional TMT proteomics marker quantitation technology, DIA boasts high stability and wide coverage; therefore, it was used in the present study to compare the non-tumorigenic MDCK cell line CL23 and the tumorigenic wild-type cell line M60. RT-qPCR and immunoblotting were used to verify the mRNA and protein levels of the screened proteins, and RNAi analysis was subsequently used to screen the effects of key genes on tumorigenesis. Our results contribute to a more comprehensive understanding of the molecular mechanism of animal cell tumorigenesis and provide a basis for the selection of target genes for the artificial construction of non-tumorigenic gene-engineered MDCK cell lines.

2. Materials and Methods

2.1. Cell culture

The MDCK adherent cell line M60 (CCL-34, ATCC) and the MDCK CL23 strain obtained from the monoclonal screening of M60 were cultured in DMEM (Lanzhou Baining Biotechnology Co., Ltd.) supplemented with 10% newborn bovine serum (Lanzhou Minhai Biotechnology Co., Ltd.) at 37, 5% CO2 in T75 culture flasks (Corning, 430641).

2.2. Cell proliferation assay

Cells were seeded onto 6-well plates at a density of $5 \times 10^3$ cells per well, with 12 groups of cells per strain in triplicate, and cultured at 37, 5% CO2. The following day, 1 mL 0.25% trypsin solution was added to three wells of each group and the plate was incubated at 37 for 10 min. Prior to complete detachment of the cells, the trypsin was removed and replaced with fresh culture medium, and a homogenous cell suspension was prepared by light pipetting. An automatic cell counter (Countstar, Allite Biotechnology (Shanghai))
Cells were counted and averaged over the three Wells, and counted every 24 hours until the number of cells no longer increased.

2.3. In vitro cell migration assay

Cells were seeded onto 6-well plates at a density of $6 \times 10^5$ cells per well and cultured at 37, 5% CO2. At 90% confluence, a flat incision was made in the central area of the cell monolayer using a 200-μL tip. Cells were carefully washed with PBS to remove exfoliated cells, which was replaced by fresh medium. Cells were observed and photographed every 4h using an inverted microscope.

2.4. Clone formation assay

Adherent cells were digested with trypsin and diluted for counting. Cells were seeded onto 6-well plates containing 3 mL prewarmed medium at a density of $1 \times 10^3$ cells per well and shaken for even dispersal. Cells were cultured at 37, 5% CO2 for 2–3 weeks. Cultures were terminated upon the appearance of macroscopic clumps of clonal cells in the wells. The supernatant was discarded and the cells were carefully washed twice in PBS. Cells were fixed in methanol for 15 min and subsequently stained with crystal violet for 10–30 min. The clonal cell clumps were counted directly by eye.

2.5. Tumorigenicity assay in nude mice

Forty BALB/c nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.), Average weight: 18g. All the animals were acclimated under standard laboratory conditions (ventilated room, 25±1 degC, 60±5 % humidity, 12 h light/dark cycle) and had free access to standard water and food (SCXK-2021-0006). All procedures were conducted in accordance with the “Guiding Principles in the Care and Use of Animals” (China) and were approved by the Laboratory Animal Ethics Committee of Northwest Minzu University (xbmt-sm-2022045). A total of $1 \times 10^7$ cells resuspended in 0.1 mL PBS were subcutaneously injected into the back of the neck of nude mice. Animals were examined for tumor formation over a 3-month period.

2.6 DIA omics

2.6.1 Protein extraction

Using a grinder (60 Hz frequency for 2 min), the appropriate amount of steel beads was added to each sample for homogenization, followed by the addition of 1 mL of 1x Cocktail containing SDS L3 and EDTA at a final concentration of 10 mM. The solution was placed on ice for 5 min and a final concentration of 10 mM DTT was added. After centrifugation at 25,000 g at 4 °C for 15 min, the supernatant was collected. DTT at a final concentration of 10 mM was added and incubated at 56 °C for 1 h. IAM with a final concentration of 55 mM was added and placed in a dark room for 45 min. Cold acetone was added to the protein solution at a ratio of 1:5 and stored at -20 °C for 30 min. The supernatant was separated by centrifugation at 25,000 g at 4 °C for 15 min and discarded. The resulting precipitate was air-dried, and an appropriate amount of SDS-free L3 was added. Protein dissolution was promoted using a grinder (60 Hz frequency for 2 min), the solution was centrifuged at 25,000 g at 4 °C for 15 min, and the resulting supernatant contained the proteins for subsequent analysis. Protein extraction quantification and quality control was performed using the Bradford assay and SDS-PAGE.

2.6.2 Proteolysis

Proteolysis was performed by adding 2.5 μg of trypsin to each 100 μg protein sample (protein:enzyme ratio = 40:1) and digested for 4 h at 37 °C. The enzymatically hydrolyzed peptides were desalted using a Strata X column and vacuum-drained.

2.6.3 RP-HPLC peptide separation at high pH

A Shimadzu LC-20AD HPLC Pump was used to mix an equal volume of peptides from each sample, which were then diluted and injected with mobile phase A (5% ACN, pH 9.8). A Gemini C18 column (4.6 × 250 mm, 5 μm) was used for liquid phase separation of the samples. Elution peaks were monitored at a wavelength...
of 214 nm with one fraction collected every minute; samples were combined with chromatographic elution peak maps to obtain 10 fractions, which were then freeze-dried.

2.6.4 DDA library construction and DIA quantitative mass spectrometry detection (Nano-LC-MS/MS)

The drained peptide samples were redissolved with mobile phase A (2% ACN, 0.1% FA), centrifuged at 20,000 g for 10 min, and the supernatant was collected and injected into the column. Separation was performed using the Thermo UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The samples were then connected in series with a self-contained C18 column (150 μm inner diameter, 1.8 μm particle size, ~35 cm column length) and separated at a flow rate of 500 nL/min through the following effective gradient: 0–5 min, 5% mobile phase B (98% ACN, 0.1% FA); 5–120 min, mobile phase B linearly increased from 5% to 25%; 120–160 min, mobile phase B increased from 25% to 35%; 160–170 min, mobile phase B increased from 35% to 80%; 170–175 min, 80% mobile phase B; 175–180 min, 5% mobile phase B. The nanoliter liquid phase separation end was directly connected to the mass spectrometer for data dependent acquisition (DDA) library construction and DIA mass spectrometry detection. Liquid phase separated peptides were ionized through a nanoESI source and inserted into a Q Exactive HF-X tandem mass spectrometer (Thermo Fisher Scientific) with DDA mode detection. Mass spectrometry and protein identification services were provided by BGI Genomics (Shenzhen, China).

2.7. Real-time fluorescent quantitative PCR

TRIzol (Thermo Fisher Scientific) reagent was used to extract total RNA, and cDNA was synthesized by reverse transcription using 50 μg cDNA as a template. Subsequently, SYBR Green fluorescence quantitative PCR was used to detect changes in gene expression. The reaction was carried out under the following conditions: pre-denaturation at 95 for 15 min, 40 cycles of denaturation at 95 for 10 s, annealing at 60 for 20 s, and extension at 72 for 30 s. Fusion curves were analyzed after amplification. The relative transcript levels of genes were calculated using the 2-t method, with GAPDH as the reference. The primers and target sequences used in this study are shown in Supplementary Table 1, 2.

2.8 Immunoblotting

Cells were washed in PBS and subsequently treated with RIPA lysis buffer (PC101) containing PMSF (GRF101) (Shanghai Yase Biomedical Technology Co., Ltd.) for 30 min. The samples were centrifuged at 12000 × g for 10 min, and the protein concentration was determined using the BCA method. Proteins were separated by 7.5% SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA). Samples were incubated with the following primary antibodies overnight at 4: anti-CDC20 (1:2000 dilution, Proteintech Group, Inc), anti-BIRC5 (1:2000 dilution, Proteintech Group, Inc), anti-SHCBP1 (1:2000 dilution, Proteintech Group, Inc), anti-TNFAIP2 (1: 2000 dilution, Proteintech Group, Inc), anti-CCNB1 (1:2000 dilution, Proteintech Group, Inc), anti-PTPN14 (1: 2000 dilution, Proteintech Group, Inc), anti-NP(1: 2000 dilution, Proteintech Group, Inc), and anti-GAPDH (1:1000 dilution, Cell Signaling). After washing with PBS, samples were incubated with an HRP (horseradish peroxidase)-conjugated anti-rabbit secondary antibody (1:1000 dilution, ZGBBT) for 1 h at room temperature. An ELC detection kit (PerkinElmer, Inc., MA) and the Tanon 5500 gel imaging system (Tanon Science & Technology Co., Shanghai, China) were used to visualize the results.

2.9. RNAi assay

MDCK M60 cells were seeded onto 12-well plates at a density of 2 x 105 per well and cultured for 24 h in DMEM supplemented with 10% fetal bovine serum and 1% peni-cillin-streptomycin. Cells were infected with lentivirus containing CDC20 shRNA or control vector at an MOI of 100 for 24 h. The lentivirus was then removed and added to the culture medium. After an additional 24 h of culture, 4 mg/mL puromycin was added to screen the cells. After five successive passages, the mRNA and protein levels of CDC20 were detected to verify knockdown. The CDC20-knockdown cells were named shCDC20, and the vector control cells were named NC-shRNA.

2.10 IAV infection and expression determination
ShRNA control and shCDC20 MDCK cells in the logarithmic growth phase were seeded onto 12-well plates at a density of 2 x 10^5 and cultured at 37, 5% CO2 to 90% confluence. The medium was then removed and the cells were washed twice with PBS. Cells were infected with A/Puerto Rico/8/34 (A/PR/8/34) H1N1 virus at an MOI of 0.01 and cultured in serum-free medium containing TPCK trypsin (2 mg/mL) for 36 h at 37, 5% CO2. Cells were collected and incubated with an anti-NP (nucleoprotein) (1:2000 dilution, Proteintech Group, Inc) primary antibody, followed by an HRP (horseradish peroxidase)-conjugated anti-rabbit secondary antibody (1:1000 dilution, ZGBBT). An ELC assay kit (PerkinElmer, Inc., MA) and a Tanon 5500 gel imaging system (Tanon Science & Technology Co. Shanghai, China) were used to visualize the results.

2.11 Statistics
Statistical analyses were performed using GraphPad Prism Software version 8.0 (Grapad Prism Software, La Jolla, CA). All data in the study are expressed as the mean +/- standard deviation of at least three independent experiments. Statistical comparisons were performed by unpaired t-tests (for two-group comparisons) or one-way ANOVA (for multiple group comparisons). P<0.05 was considered statistically significant.

2.12 Data Availability Statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

3. Results
3.1. The proliferation and migration rates of selected monoclonal CL23 cells were significantly decreased
In our previous study, we found that the proliferation and migration abilities of CL23 cells were significantly decreased. The results of the absolute cell growth number experiment show that CL23 had a significantly reduced proliferation rate in comparison with M60 cells (Fig. 1A). Analysis of the migration properties of the cell lines by a scratch healing assay (Fig. 1B) shows that M60 cells displayed rapid cell migration within 24 h, completely healing the gap. By contrast, CL23 cells moved toward the center of the scratch as an island protrusion, and the gap had not healed after 24 h. Moreover, the clone formation assay shows that the number and area of CL23 colonies were significantly reduced in comparison with M60 cells, indicating that cell adhesion and proliferation activities were significantly reduced (Fig. 1C). These data demonstrate that the proliferation, migration, and colony formation rates of CL23 cells were significantly lower than those of M60 cells.
3.2 CL23 cell line had no tumor formation in nude mice

To evaluate the tumorigenic ability of M60 and CL23 cells, an in vivo tumor formation assay was performed in nude mice. Cells were subcutaneously injected into the back of the neck of Athymic nude mice (10 per group) at a density of $1 \times 10^7$. Three months after injection, M60 cells had formed tumors at the injection site of all 10 nude mice (Fig. 2A), reaching a volume greater than 1 cm³; however, no subcutaneous tumor formation was observed in any of the 10 nude mice injected with CL23 cells (Fig. 2B). These results demonstrate that CL23 cells lost the tumorigenic phenotype of wild-type MDCK cells.
3.3. MDCK cell tumorigenesis-related proteins

To further explore the key proteins and possible molecular mechanisms underlying the tumorigenicity of MDCK cells, the differentially expressed proteins between M60 and non-tumorigenic monoclonal CL23 cells were assessed using proteomics. The MSstats software was used to perform the error correction and normalization of each sample in the system. A fold change $>2$ and a P value $<0.05$ were used as the screening criteria for significant differential expression. A total of 4,898 proteins were quantitatively detected between M60 and CL23 cells in all samples. In comparison with M60 cells, 912 significantly differentially expressed proteins were identified in CL23 cells, of which 422 were upregulated and 490 were downregulated (Fig. 3A).

It can be seen that there are significant differences between M60 and CL23 proteins, and the expression pattern of differential proteins among the three biological replicates of the same cell type is the same. The differentially expressed proteins had a good correlation with the differences in tumorigenic phenotypes between cells (Fig. 3B). To screen out key proteins affecting the tumorigenicity of MDCK cells, GO, KEGG, and Uniprot analyses were performed with a view to finding involvement in cell proliferation, cell cycle regulation, apoptosis, and invasion. GO enrichment analysis of the 912 differentially expressed proteins between M60 and CL23 cells demonstrates that the signaling pathways were concentrated in cell growth and death, signal transduction, replication and repair, cancer, and the endocrine system (Fig. 3C). The pathway map of significantly differentially expressed proteins (Fig. 3D) shows that there was a large number of these proteins, all of which were enriched in cell cycle signaling, MicroRNAs signaling in cancer, and apoptosis signaling. Cell cycle changes are regulated by a series of signal transduction pathways; therefore, among the proteins related to MDCK tumorigenicity, those regulating the cell cycle attracted our attention. At the same time, the ability of cells to resist apoptosis plays an important role in the generation and development of cancer. KEGG signaling pathway analysis shows that the differentially expressed proteins were closely related to the apoptosis pathway, which suggests that subsequent studies need to focus on genes related to apoptosis.

3.4. Validation of mRNA expression levels of the screened genes

High-throughput omics analysis can contain errors or false positives; therefore, RT-qPCR was used to verify the differential expression of the screened genes at the mRNA level. The results show that a total of 15...
significantly differentially expressed proteins were present in CL23 cells as compared with M60 cells (Table 1), of which 11 were significantly downregulated, including ARG2, TNFAIP2, BIRC5, CDC20, PCNA, CSE1L, FIGNL1, SHCBP1, and CCNB1. Four proteins in CL23 cells were significantly upregulated, namely EPHA2, NDGR1, ZFP36, and PTPN14, of which PTPN14 was upregulated by the largest fold (Fig. 4).

To further characterize the possible relationships among the screened tumorigenesis-related proteins, a protein–protein interaction network was constructed using the STRING database version 9.0 (Fig. 4B, C). The majority of the screened proteins may be involved in direct or indirect interactions, among which SHCBP1, CDC20, and CCNB1 are located at the core of the interaction network. CDC20 and CCNB1 are both cyclin proteins, whose overexpression is associated with the activation of CDC2 kinase, which can override p53-mediated G2/M arrest [10]. As a spindle-associated protein, SHCBP1 is also related to the cell cycle; therefore, the interaction between CDC20 and SHCBP1 deserves further attention in subsequent studies.

3.5. Validation of the protein expression levels of the screened genes

Six proteins that interact with CDC20 and have the most significant differences in expression levels between M60 and CL23 cells were selected: CDC20, BIRC5, CCNB1, SHCBP1, TNFAIP2, and PTPN14. The changes in protein expression levels were further verified by immunoblotting as shown in Fig. 6. In comparison with M60 cells, the protein expression levels of CDC20, BIRC5, CCNB1, SHCBP1, and TNFAIP2 were significantly downregulated in CL23 cells, and that of PTPN14 was significantly upregulated. These results are consistent with those of the iTRAQ and RT-qPCR.
3.6. Knockdown of CDC20 significantly inhibits the proliferation and migration of MDCK cells

CDC20 is a key activator of the pro-mutation complex (APC/C) and plays an essential role in mitotic regulation, being a positive regulator of cell division and an important cyclin. The expression of CDC20 is significantly upregulated in a variety of cancers and is one of the important factors affecting the tumorigenicity of cells, lying at the core of the protein–protein interaction network in the present study. Accordingly, three shRNA targeting the specific sequence of CDC20 were designed and transferred into MDCK cells by a
lentiviral expression vector system to achieve CDC20 gene knockdown. Both mRNA and protein expression levels of CDC20 were significantly reduced in the two target shCDC20 cell lines in comparison with the vector control cells (NC-shRNA cells) (Fig. 6). To further verify the effect of CDC20 knockdown on tumorigenesis, in vitro tumorigenesis-related phenotypic analysis was performed. The results show that the proliferation, migration, and clonicity of shCDC20 cells were significantly lower than those of NC-shRNA cells (Fig. 7A–C), suggesting that CDC20 plays a role in promoting tumorigenesis in MDCK cells and can be used as an effective target gene for the inhibition of tumorigenesis. Since the MDCK cell line is an important matrix for influenza vaccine production, the yield of virus is also a concern. To further verify whether CDC20 knockdown affects virus replication, the expression of viral NP was assessed in shCDC20 and NC-shRNA cells (Fig. 7D). The expression of NP protein in shCDC20 cells was higher than that in NC-shRNA cells, indicating that influenza virus replication was enhanced in shCDC20. MDCK cells derived from shCDC20 could be used as a good vaccine matrix cells.

Table 1. Fifteen proteins that differ significantly between high and low tumorigenic MDCK cells

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4. Discussion

In the era of COVID-19 globalization and seasonal outbreaks of influenza, it is necessary to use animal cells as a matrix to produce vaccines. Currently, influenza vaccines produced using MDCK cells have been marketed and widely administered in many countries around the world; however, the safety of the vaccine remains controversial due to the potential for tumor formation. Screening of a large amount of monoclonal tumor cell lines, CL23, indicates that H1N1 influenza virus proliferation was not significantly altered in comparison with that in wild-type M60 cells. Preliminary evaluation of this established cell line is of great importance for improving the biological safety of MDCK cell-derived influenza vaccines. Moreover, it provides the opportunity to explore genes that are involved in the tumorigenesis of animal cells and reveal the underlying molecular mechanisms.

The tumorigenic ability of different cells is controlled by different molecular mechanisms and signaling pathways, and the extent of angiogenesis typically determines the volume and growth rate of tumor tissue. Previous studies have shown that BIRC5 can promote the migration and invasion of tumor cells and regulate the expression of angiogenesis-related factors[11]. In the present study, we found that the expression of BIRC5 was significantly higher in M60 cells than in CL23 cells, which is consistent with the results of in vivo tumorigenesis experiments. We hypothesize that BIRC5 plays a role in promoting angiogenesis and tumor growth in M60 cells. At the same time, tumorigenicity is also affected by a variety of signaling pathways, among which is the well-studied Wnt/β-catenin signaling pathway. The Wnt signal transduction pathway is inactive in normal mature cells and only activated during embryonic development, participating in a variety of developmental patterns[12]. Mutations in oncogenes, tumor suppressor genes, or components of the Wnt signal transduction pathway (such as APC, β-catenin, and axin) lead to inappropriate activation, which is related to the occurrence and development of tumors[13]. Previous studies have demonstrated that overexpression of TNFAIP2 (tumor necrosis factor-α-induced protein 2) promotes the proliferation and metastasis of esophageal cancer by activating the Wnt/β-catenin signaling pathway[14]. Our experiments show that TNFAIP2 expression in M60 cells was significantly higher than that in CL23 cells. The tumorigenicity of M60 cells was confirmed by animal experiments. We hypothesize that TNFAIP2 acts through Wnt/β-catenin to promote M60 cell tumorigenesis. Moreover, the cell cycle is an important factor affecting cell apoptosis; therefore, various proteins that regulate the cell cycle have attracted increasing attention. Studies have shown that

CCNB1, an important member of cyclin family, is a key ini-tiator and rigorous quality control step of mitosis. It has a piv-otal role in regulating cyclin-dependent kinase 1 (CDK1) and forming complex with itand promoting the transition from G2 phase to mitosis[15]. Here, we demonstrate that CCNB1 was significantly upregulated in M60 cells as compared with CL23 cells. According to the STRING network, CCNB1 interacts with CDC20; therefore, we hypothesize that these proteins jointly regulate the cell cycle in M60 cells to mediate apoptosis and promote tumorigenesis.

Our data show that CDC20 was highly expressed in M60 cells and centrally located in the STRING network. Following knockdown of CDC20 by RNA interference technology, the proliferation, migration, and colony formation abilities of M60 cells were greatly reduced; therefore, we hypothesize that CDC20, as a protein affecting the cell cycle, promotes tumorigenicity of M60 cells. Other studies have reported that CDC20 is highly expressed in a large number of cancer cells, and inhibition of CDC20 expression in HCC cells delays the progression of hepatocyte mitosis and inhibits proliferation[16]. CDC20 plays a role in promoting cancer cell growth through several signaling pathways. In cutaneous squamous cell carcinoma, downregulation of CDC20 inhibits cell proliferation, induces cell cycle arrest, promotes cell apoptosis, and reduces cell migration ability by inhibiting the Wnt/β-catenin signaling pathway[17]. The Wnt/β-catenin signaling pathway plays a crucial role in regulating cell growth, cell development, and normal stem cell differentiation. Constitutive activation of Wnt/β-catenin signaling is found in many human cancers. Epithelial–mesenchymal transition
is an important process in tumor development, for which the accumulation of β-catenin in the nucleus is required; therefore, the effect of CDC20 on the Wnt/β-catenin signaling pathway is worthy of attention.

In addition to its involvement in the Wnt/β-catenin signaling pathway, CDC20 also plays an important role in the Notch signaling pathway. Overexpression of Notch-1 in sarcoid osteoma cells enhances the expression of CDC20. Moreover, CDC20 overexpression alleviates the inhibitory effects of Notch-1 knockdown on sarcoid osteoma cell viability, migration, and invasion\cite{18}. Notch signaling plays a key role in a variety of biological processes including cell proliferation and apoptosis\cite{19}. Further, a large body of evidence indicates that the Notch signaling pathway is involved in the pathogenesis and development of cancer through a variety of mechanisms, especially in the generation of solid tumors\cite{20}. The molecular mechanism and location of CDC20 in Notch signaling require further exploration.

In addition to promoting cell growth, CDC20 also inhibits apoptosis. CDC20 targets Bim, the pro-apoptotic protein of BH3, for ubiquitination and destruction, thus inhibiting cell apoptosis. Depletion of CDC20 enhances cell sensitivity to apoptotic stimuli\cite{21}; therefore, CDC20 plays an important role in tumor formation and development, which needs further study. As a cell line for vaccine production, great attention is paid to the virus replication rate in CL23 cells. Our experiments show that knockdown of CDC20 did not inhibit the replication of influenza A H1N1 virus; thus, CDC20 can be used as a target gene for genetic engineering of low-tumorigenic MDCK cells.

In current vaccine research and development, the search for stromal cells without tumorigenicity remains ongoing. Here, we successfully screened differentially expressed proteins between non-tumorigenic and highly tumorigenic cell lines using proteomics and verified these at the mRNA and protein levels. Knockdown cell lines were also verified to possess reduced proliferation, migration, and colony formation phenotypes.

It is promising to be able to screen differentially expressed proteins and construct low-tumorigenic cell lines by genetic engineering; however, the process of tumor formation requires multiple genes and pathways, and the synergistic effects of other molecules and signaling pathways must be explored in the future. Our study lays a theoretical foundation for the construction of genetically engineered cell lines incapable of forming tumors for the safe production of vaccines in the future.

5. Author Contributions

Xiaoming Yang, Zhenbin Liu provided the idea of the experiment, Jiamin Wang, Dongwu Jin, Zilin Qiao provided the experimental materials and venues, Zhenbin Liu, Mengyuan Fei designed and conducted the experiment. Mengyuan Fei, Geng Liu, Zhenyu Qiu, Siya Wang analyzed DIA omics, tumorigenic protein mRNA expression, and protein expression data, and prepared the manuscript. Kai Duan, Xuanxuan Nian, and Siya Wang visually analyzed the experimental results. Xiaoming Yang, Zhongren Ma supervised and led the experiment. All authors participated in manuscript preparation. Xiaoming Yang provided full oversight of the study.

6. Funding

This research was funded by The National Natural Science Foundation of China, grant number 32160164
This research was funded by The Natural Science Foundation of Gansu Province, grant number 18JR2JA004
This research was funded by The Fundamental Research Funds for the Central Universities, grant number(31920210033, 31920210002)

7. Institutional Review Board Statement

All procedures were conducted in accordance with the “Guiding Principles in the Care and Use of Animals” (China) and were approved by the Laboratory Animal Ethics Committee of Northwest Minzu University(xbmu-sm-2022045).

8. Data Availability Statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

9. Conflicts of Interest

The authors declare no conflict of interest

10. Supplementary tables

Table 1. Differential protein primer synthesis sequence

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<th>Primer name</th>
<th>Forward primer</th>
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<td>ggCAgCggACCATATTCCA</td>
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Table 2. shRNAsynthesize oligo information

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